PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of

Valerie CHEYNET-SAUVION et al.

Application No.: 09/402,131

Filed: December 8, 1999

Group Art Unit: 1655

Examiner: B. Sisson

Docket No.: 104458

For: RNA-DEPENDENT RNA POLYMERASE FUNCTIONING PREFERABLY ON RNA MATRIX AND PROMOTER-DEPENDENT TRANSCRIPTION PROCESS WITH SAID RNA-DEPENDENT RNA POLYMERASE

AMENDMENT

Director of the U.S. Patent and Trademark Office
Washington, D.C. 20231

Sir:

In reply to the Office Action mailed December 5, 2000, please amend the above-identified patent application as follows:

IN THE SPECIFICATION:

Page 30, line 5 to page 31, line 24, delete current paragraph and insert therefor:

The reactions are performed in 20 µl of a buffer derived from that described by J.F. Milligan, D.R. Groebe, G.W. Witherell, O.C. Uhlenbeck, Nucleic Acids Res. 25, 8783 (1987), namely Tris-HC1 40 mM, pH 8.1, spermidine 1 mM, PEG 8% (g/V), TRITON (a surfactant) 0.01% (V/V), BSA 5 µg/100 µl, 1 µl (40 u) of porcine RNAguard (Pharmacia Biotech), UTP 12.5 µM, a 32P UTP 0.5 µCi (Amersham, 10 mCi/ml 400 Ci/mmol) 0.4 mM of the three ribonucleoside triphosphates A, G, C, $Mg(OAc)_2$ 6 mM. The template concentration is set at 10^{11} copies of each strand in 20 µl of reaction. The wild-type T7 RNA polymerase is used at 0.5 µM (100 ng/20 µl), the mutated T7 RNA polymerase R627A at

5.65 μM (730 ng/20 μl). Before adding the enzymes, the reactions are denatured for 5 minutes at 65°C in a heating block and then gradually brought to 37°C. The reactions are initiated by the addition of the polymerases, incubated for 1 hour at 37°C and then stopped by the addition of an equal volume of 2× blue formamide (formamide 90%, EDTA 25 mM, xylene cyanol 0.02%, bromophenol blue 0.02%) and denatured for 5 minutes at 95°C. 20 μl of each reaction are deposited on a denaturing gel (20% acrylamide, urea 7 M, 1X TBE), and then after migration, the gel is autoradiographed at -70°C on a Biomax MR film (Kodak). The results (electrophoretic profiles) are presented in Figure 5, and in particular the transcription results obtained with the mutated T7 RNA polymerase R627A (wells 1-3) and the wild-type T7 RNA polymerase (wells 4-6), on the single-stranded RNA templates (wells 1 and 4), double-stranded DNA (wells 2 and 5), and single-stranded DNA (wells 3 and 6). The transcription on single-stranded RNA, detected by detection of a complete transcript of 33 bases, is possible using the mutated T7 RNA polymerase R627A (well 1) and not the wild-type enzyme (well 4) which produces on the other hand many abortive transcripts; see nevertheless the different results obtained in Example 3 below. The mutated T7 RNA polymerase R627A exhibits a residual transcription activity on double-stranded DNA (well 2), characterized by the presence of a predominant transcript which is smaller in size than the expected transcript, and the presence of a small quantity of abortive products. On single-stranded DNA (well 3), this transcript of abnormal size disappears, whereas the quantity of abortive products increases. By contrast, the wild-type enzyme allows the production of specific transcripts in the presence of DNA templates (wells 5 and 6), this enzyme exhibiting, moreover, a better transcription activity on the double-stranded DNA template (well 5) than on the single-stranded DNA template (well 6); for these two templates, the wild-type enzyme induces the synthesis of numerous abortive transcripts. These results show that the replacement of the arginine 627 by an alanine confers on the mutant enzyme the

possibility of synthesizing RNA from an RNA template and induces the loss of capacity to synthesize RNA from a DNA template.

C Cont.

REMARKS

Claims 35-68 are pending. Claims 48-68 are withdrawn from consideration. The specification is amended herein. The attached Appendix includes a marked-up copy of the rewritten paragraph (37 C.F.R. 1.121(b)(1)(iii)).

The Office Action objects to the Declaration as being defective because non-initialed and/or non-dated alterations have been made to the Declaration citing 37 C.F.R. §1.52(c), and because it was allegedly not executed in accordance with 37 C.F.R. §1.66 or §1.68. Applicants respectfully traverse the assertion that the Declaration is defective. First, there is no requirement that the Declaration be executed in cursive handwriting, as is suggested in the Office Action. The Declaration can be executed in any form that one normally uses to denote one's signature, including by printing one's name. In addition, since the Declaration is executed on the same pages as it is altered, the alterations made on the Declaration are signed and dated on the same sheet of paper, as required by 37 C.F.R. §1.52(c).

For all of these reasons, it is believed that the prior Declaration is acceptable. Therefore, the objection should be reconsidered and withdrawn.

The specification is objected to based on the use of the trademark TRITON in an improper format. The specification has been amended in order to correctly identify the trademark. Therefore, the objection should be reconsidered and withdrawn.

Claims 35-47 are rejected under 35 U.S.C. §112, first paragraph, for allegedly lacking enablement. Applicants respectfully traverse the rejection.

The present invention is based on the discovery of a new use for a known family of RNA polymerases, which were known to have a capacity for transcribing a double-stranded template of DNA, i.e., for synthesizing an RNA sequence complementary to one of the

strands of the DNA template. It has been discovered that the family of RNA polymerases (RNAPs) also has the capacity for transcribing an RNA template, i.e., for synthesizing an RNA sequence complementary to the RNA template. It has further been discovered that by mutating the RNAPs, mutated RNAPs can be easily obtained that are capable of synthesizing a transcriptional product from an RNA template with a better yield than from a DNA template.

The family of RNAPs that can be used according to the present invention is one that is capable of transcribing under the control of a promoter. The promoters of the family have a consensus sequence from position -17 to position -1. See the specification, at page 5, lines 18-23. This family includes the RNA polymerases of phages T3, T7 and SP6. The fact that there is high similarity between these RNAPs is shown, for example, by the fact that substitution of a single amino acid in the T3 enzyme allows the mutated enzyme to specifically recognize the T7 RNAP promoter and vice versa. See the specification at page 14, lines 17-21. Similar exchanges of specificities have also been found between the T7 and SP6 polymerases. See W.T. McAllister (copy attached), at page 388, left column, lines 14-18. In addition, all of the phage RNAPs are considered by those skilled in the art as "T7-like RNA polymerases." See, for example, M. Chamberlin and T. Ryan in The Enzymes, Vol. XV, Chapter 4, pages 87-91 (copy attached), at, in particular, page 87, heading II; page 88, line 4; page 89, heading II; page 90, second full paragraph, citing T3 RNA polymerases; and page 91, lines 3-4, citing SP6 RNA polymerase.

Example 2 of the present application shows that a mutated T7 RNAP can transcribe an RNA template. Example 3 further shows that the wild-type T7 RNAP can also transcribe an RNA template. Thus, although T7 RNA polymerase is a DNA-dependent RNA polymerase, as noted in the Office Action, this polymerase also has an RNA-dependent RNA polymerase activity. Thus, the recitation of an RNA-dependent RNA polymerase activity is not a

typographical error, as suggested in the Office Action. Instead, as shown in the present application, polymerases known to transcribe DNA can also be used by the present method to transcribe RNA.

In view of the well recognized similarity of the various phage RNAPs having a promoter with a consensus sequence from -17 to -1, it is respectfully submitted that the claims of the present application constitute a moderate and legitimate generalization of the results reported in the examples. More particularly, it is quite easy for those skilled in the art to use other RNAPs and to produce and use mutated RNAPs and to verify by routine experiments, as is reported in the examples of the present specification, whether the enzymes can transcribe an RNA template.

For all of the above reasons, it is respectfully submitted that claims 35-47 are enabled by the present application. Therefore, the rejection under 35 U.S.C. §112, first paragraph, should be reconsidered and withdrawn.

In view of the above amendments and remarks, it is respectfully submitted that the present application is in condition for allowance. Favorable consideration and prompt allowance are therefore respectfully requested.

Should the Examiner believe that anything further would be required in order to place the application in better condition for allowance, the Examiner is invited to contact Applicants' undersigned representative at the telephone number listed below.

Respectfully submitted,

William P. Berridge
Registration No. 30,024

Melanie L. Mealy
Registration No. 40,085

WPB:MLM/jca

Attachments:
Appendix
McAllister
Chamberlin et al.

Date: June 5, 2001

OLIFF & BERRIDGE, PLC
P.O. Box 19928
Alexandria, Virginia 22320
Telephone: (703) 836-6400

DEPOSIT ACCOUNT USE AUTHORIZATION
Please grant any extension necessary for entry;
Charge any fee due to our Deposit Account No. 15-0461

APPENDIX

Changes to Specification:

Page 30, line 5 to page 31, line 24:

The reactions are performed in 20 µl of a buffer derived from that described by J.F. Milligan, D.R. Groebe, G.W. Witherell, O.C. Uhlenbeck, Nucleic Acids Res. 25, 8783 (1987), namely Tris-HC1 40 mM, pH 8.1, spermidine 1 mM, PEG 8% (g/V), TRITON (a surfactant) ~~triton~~ 0.01% (V/V), BSA 5 µg/100 µl, 1 µl (40 u) of porcine RNAguard (Pharmacia Biotech), UTP 12.5 µM, a 32P UTP 0.5 µCi (Amersham, 10 mCi/ml 400 Ci/mmol) 0.4 mM of the three ribonucleoside triphosphates ~~triphoshates~~ A, G, C, $Mg(OAc)_2$ 6 mM. The template concentration is set at 10^{11} copies of each strand in 20 µl of reaction. The wild-type T7 RNA polymerase is used at 0.5 µM (100 ng/20 µl), the mutated T7 RNA polymerase R627A at 3.65 µM (730 ng/20 µl). Before adding the enzymes, the reactions are denatured for 5 minutes at 65°C in a heating block and then gradually brought to 37°C. The reactions are initiated by the addition of the polymerases, incubated for 1 hour at 37°C and then stopped by the addition of an equal volume of 2× blue formamide (formamide 90%, EDTA 25 mM, xylene cyanol 0.02%, bromophenol blue 0.02%) and denatured for 5 minutes at 95°C. 20 µl of each reaction are deposited on a denaturing gel (20% acrylamide, urea 7 M, 1X TBE), and then after migration, the gel is autoradiographed at -70°C on a Biomax MR film (Kodak). The results (electrophoretic profiles) are presented in Figure 5, and in particular the transcription results obtained with the mutated T7 RNA polymerase R627A (wells 1-3) and the wild-type T7 RNA polymerase (wells 4-6), on the single-stranded RNA templates (wells 1 and 4), double-stranded DNA (wells 2 and 5), and single-stranded DNA (wells 3 and 6). The transcription on single-stranded RNA, detected by detection of a complete transcript of 33 bases, is possible using the mutated T7 RNA polymerase R627A

(well 1) and not the wild-type enzyme (well 4) which produces on the other hand many abortive transcripts; see nevertheless the different results obtained in Example 3 below. The mutated T7 RNA polymerase R627A exhibits a residual transcription activity on double-stranded DNA (well 2), characterized by the presence of a predominant transcript which is smaller in size than the expected transcript, and the presence of a small quantity of abortive products. On single-stranded DNA (well 3), this transcript of abnormal size disappears, whereas the quantity of abortive products increases. By contrast, the wild-type enzyme allows the production of specific transcripts in the presence of DNA templates (wells 5 and 6), this enzyme exhibiting, moreover, a better transcription activity on the double-stranded DNA template (well 5) than on the single-stranded DNA template (well 6); for these two templates, the wild-type enzyme induces the synthesis of numerous abortive transcripts. These results show that the replacement of the arginine 627 by an alanine confers on the mutant enzyme the possibility of synthesizing RNA from an RNA template and induces the loss of capacity to synthesize RNA from a DNA template.

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Original Contribution

STRUCTURE AND FUNCTION OF THE BACTERIOPHAGE T7 RNA POLYMERASE (OR, THE VIRTUES OF SIMPLICITY)

WILLIAM T. MCALLISTER

Department of Microbiology and Immunology, Morse Institute of Molecular Genetics, SUNY Health Science Center, Box 44, 450 Clarkson Ave., Brooklyn, NY 11203-2098, USA

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Abstract—A consideration of the properties of a number of mutants of T7 RNA polymerase, together with emerging structural information (Sousa et al., 1993) allows an interpretation of the the mechanics of transcription by this relatively simple RNA polymerase. Evidence indicating features in common with other nucleotide polymerases (such as DNA polymerases and reverse transcriptases) is reviewed.

Keywords—DNA polymerase, Reverse transcriptase, Promoter structure

INTRODUCTION

Unlike the multisubunit DNA-dependent RNA polymerases (RNAPs) of eukaryotic cells and bacteria, the RNAPs that are encoded by bacteriophage T7 and its relatives consist of a single species of protein that is capable of accurate transcription in the absence of any apparent need for auxiliary transcription factors (Chamberlin and Ryan, 1983). The striking simplicity of this transcription system makes it ideally suited for studies of RNA polymerase structure and function. The gene that encodes the phage RNAP has been cloned and may be overexpressed in bacterial cells, allowing genetic and biochemical manipulation of the enzyme (Davanloo et al. 1984). Importantly, T7 RNAP has now been crystallized, and a number of mutants that are altered in the transcription cycle have been characterized (Bonner et al., 1992; Patra et al., 1992; Gross et al., 1993; Sousa et al., 1993).

In our work, we have taken the approach of isolating or engineering T7 RNAP mutants with defined biochemical defects and asking whether these defects can be correlated with structural information so as to interpret the mechanism of transcription. We have identified important functional domains in the RNAP, and have found that the phage RNAP exhibits interesting structural and functional homologies to other simple nucleotide polymerases, such as DNA polymerases and reverse transcriptases. Although no extended sequence homologies exist between the phage RNAPs and the multisubunit RNAPs, there are intriguing clues that suggest a relationship between the phage enzymes and certain subunits of the more complex RNAPs. Studies of this class of RNAP will, therefore, contribute significantly to our understanding of nucleotide polymerization.

MATERIALS AND METHODS

Transcription reactions

Mutant RNAP have been previously described (Gross et al., 1993); the designation *ins*xxx indicates a linker insertion mutation that lies within or immediately preceding codon xxx. All transcription reactions were carried out in a volume of 10 μl containing: 20 mM Tris-HCl (pH 7.9), 8 mM $MgCl_2$, 2 mM spermidine-HCl, 1 mM dithiothreitol, 0.5 mM each of ATP, GTP, CTP, and UTP (Pharmacia, Ultrapure), and 1 μl cell extract (Gross et al., 1993). The products were resolved by electrophoresis in 20% polyacrylamide gels followed by autoradiography (ibid).

RESULTS

Enzyme domains involved in promoter recognition

T7 RNAP is the prototype of a class of single-subunit DNA-dependent RNAPs that includes the

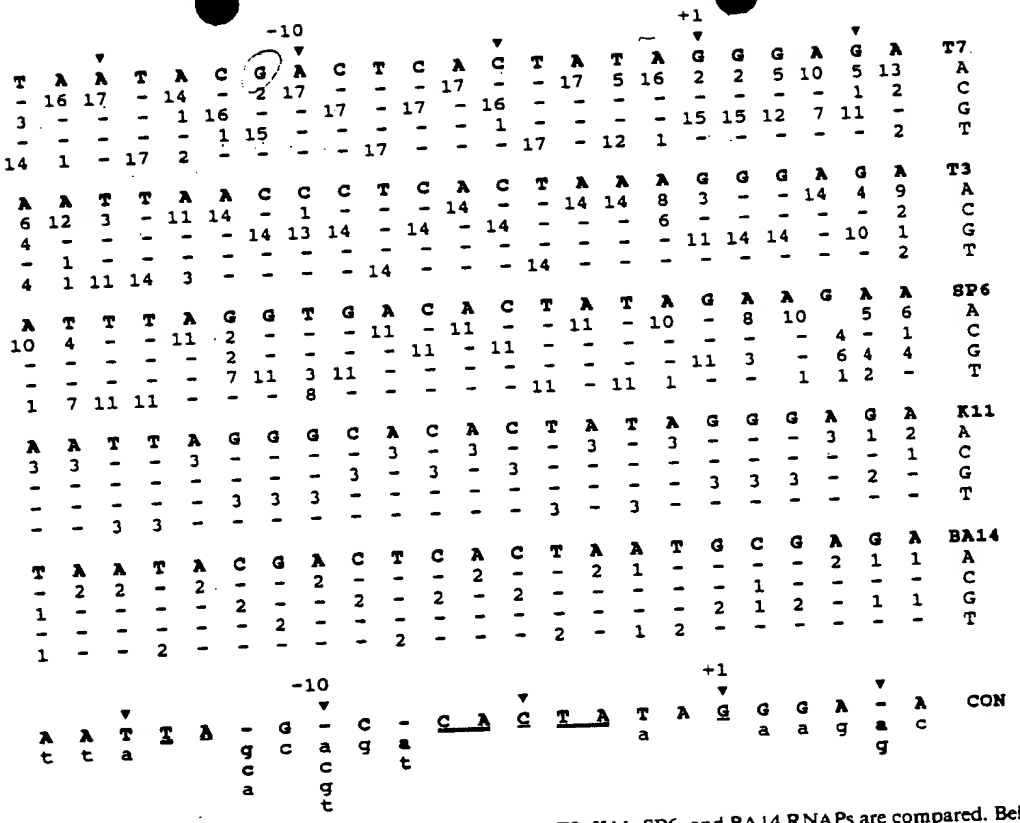

Fig. 1. Structure of phage promoters. Promoter sequences for T7, T3, K11, SP6, and BA14 RNAPs are compared. Below each consensus promoter is a summary of the frequency of each basepair in 17 natural T7 promoters, 14 T3 promoters, 11 SP6 promoters, 3 K11 promoters, and 2 BA14 promoters (Dunn and Studier, 1983; Brown et al., 1986; Beck et al., 1989; Dietz et al., 1990; Rush, personal communication; Hausmann, personal communication). The sequence of the nontemplate strand is shown; the start site of transcription is at +1. CON represents a common sequence of the five phage consensus promoters. Upper case, double underlined letters represent a bp that is conserved in all 47 promoters; upper case single underlined letters represent a bp that is conserved in all five consensus sequences; uppercase letters represent a conserved bp in at least three out of five consensus sequences; a dash (-) indicates a position that is not conserved. Lower case bold letters below the CON line indicate alternative bps that occur twice in the five consensus sequences; lower case letters indicate an alternative bp that occurs once.

RNAPs of related phages such as T3, SP6, and K11, as well as the mitochondrial RNAPs, and potentially, a chloroplast RNAP (for review, see McAllister and Raskin, 1993). Although the other phage RNAPs are closely related by sequence homology to T7 RNAP, each phage RNAP exhibits its own characteristic specificity. A comparison of the sequences of the phage promoters reveals a common 23 bp consensus sequence that extends from −17 to +6, with initiation at +1 (see Fig. 1).

A variety of biochemical and genetic experiments support the notion of two functional domains in the promoter—a binding domain that extends from −17 to −6, and an initiation domain that extends from −6 to +6 (see Fig. 2). All of the promoters share the same sequence from −6 to +1, indicating a common function for this region of the promoter. However, the sequences of the phage promoters differ significantly in the region from −9 to −12, suggesting that discrimination of specific promoter types may rely upon differences in this region. Mutations in the binding region have been observed to reduce the affinity of the polymerase for the promoter without having a great effect on the rate of initiation, whereas mutations in the initiation region have minor effects on the binding affinity but a greater effect on the rate of initiation (Chapman and Burgess, 1987; Chapman et al., 1988). Upon binding of the RNAP to the promoter, the DNA in the initiation region becomes melted open, as evidenced by a hypochromic shift and hypersensitivity of the nontemplate (NT) strand in this region to attack by single-strand specific endonucleases (Muller et al., 1989; Osterman and Coleman, 1981).

Base modification experiments indicate that a number of important contacts between the polymerase and the promoter are made within the major

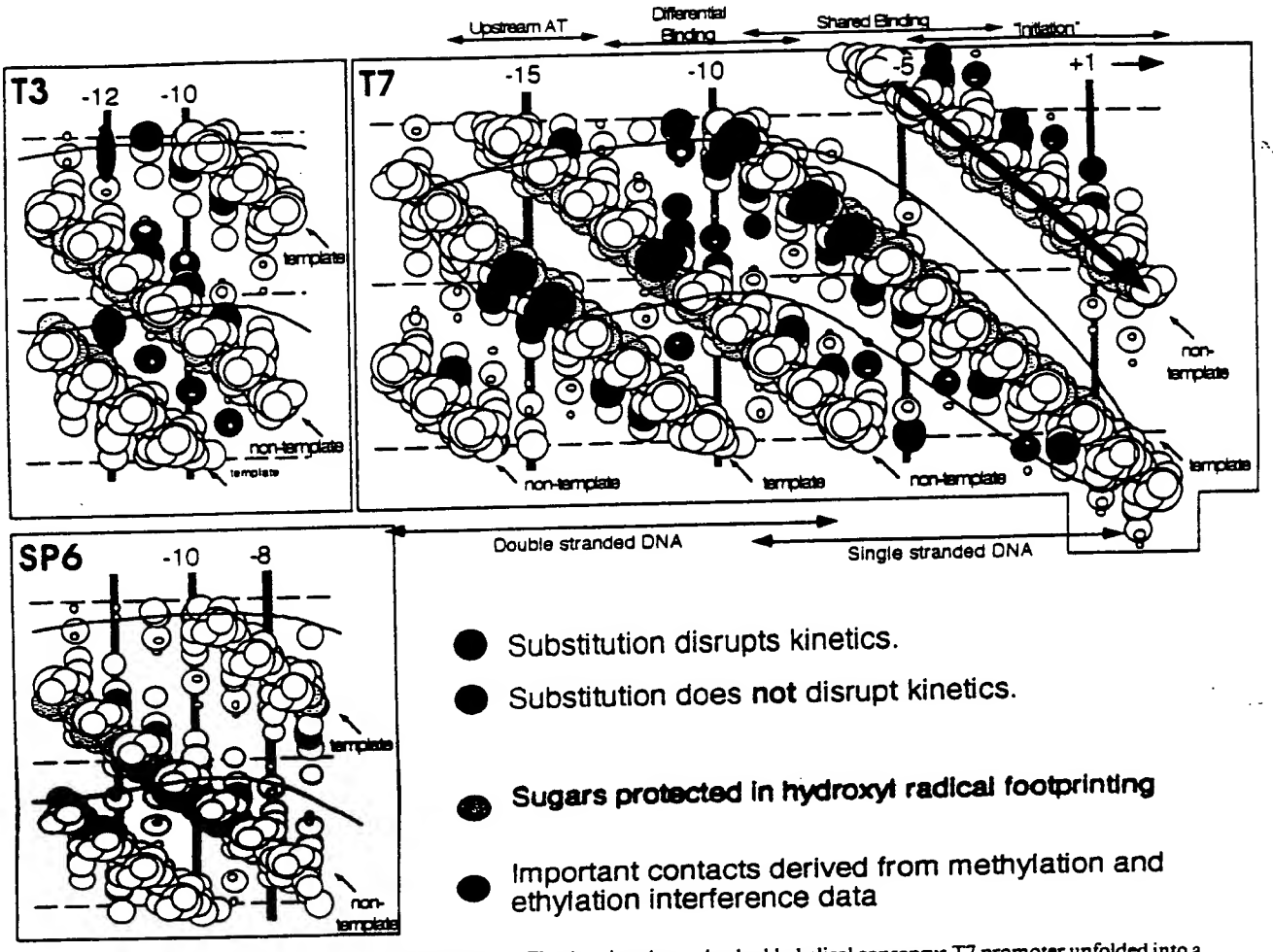

Fig. 2. Topography of RNA polymerase contacts. The drawing shows the double-helical consensus T7 promoter unfolded into a planar view; template, and nontemplate strands are indicated. Important structural elements in the promoter include: positions at which substitutions with modified bases affect the kinetics of initiation (Maslak et al., 1993; Schick and Martin, 1993), positions at which the sugar-phosphate backbone of the DNA is protected by polymerase binding as revealed by hydroxy-radical footprinting (Muller et al., 1989); and positions at which ethylation of the phosphate or methylation of the bases interferes with polymerase binding (Jorgensen et al., 1991). From these data, the contacts of the RNAP appear to involve major groove groups from −6 to −12, and minor groove contacts in the flanking regions on either side. Regions of the promoter that remain double stranded or are rendered partially single stranded during polymerase binding are indicated at the bottom (Osterman and Coleman, 1981; Muller et al., 1989). Other important regions such as the upstream AT-rich region, the region that is involved in promoter discrimination by individual RNAPs, the region in which the promoter sequences are highly conserved (shared binding), and the initiation region, are indicated at the top. Similar data for the T3 and SP6 promoters and their RNAPs are indicated in the side panels. Graphics were kindly provided by Dr. Craig Martin (University of Massachusetts).

groove and the flanking regions from bps −12 to −9, and it has been shown that the primary determinants of T3 vs. T7 promoter specificity are the bps at positions −11 and −10 (Jorgensen et al., 1991; Muller et al., 1989; Klement et al., 1990; Raskin et al., 1992). Substitution of these two bps in the T7 promoter with the corresponding bps found in the T3 promoter prevents recognition by T7 RNAP and simultaneously enables recognition by T3 RNAP (ibid).

To localize the region of the phage RNAP that is responsible for discrimination of these base pairs, hybrid T7/T3 RNAPs were constructed (Joho et al., 1990). In this way, the specificity determinant was localized to an 80 amino acid interval between residues 674 and 752. Within this interval the T7 and T3 RNAP amino acid sequences differ at only 11 positions. Site-directed mutagenesis of this region of the T7 RNAP indicates that a single amino acid is respon-

sible for discrimination of the −10 and −11 bps; when this residue (Asn) is substituted by the corresponding residue found in the T3 RNAP (Asp), the resulting mutant enzyme (T7-N748D) exhibits T3 promoter specificity, particularly for the bps found at −10 and −11 (Raskin et al., 1992). A consideration of the hierarchy of preference for each of the possible base pair combinations at −10 and −11 indicates that N748 makes direct contacts with bases on the nontemplate strand in a bidentate configuration (Diaz et al., 1993; Raskin et al., 1992). This interpretation is consistent with all of the genetic and biochemical data described above.

Substitution of other amino acids at position 748 has generated a collection of T7 RNAP mutants with altered specificities. Some of the mutant enzymes have specificities that correspond to those found in other phage RNAPs (e.g., the SP6 and K11 RNAPs), but others exhibit novel specificities not previously observed (Raskin et al., 1993). The location of residue N748 within the crystal structure of T7 RNAP is within a putative DNA binding cleft, at a position that would lie approximately one helical turn (35 Å) upstream from what is believed to be the active site (Sousa et al., 1993); see Fig. 3, and discussion below). This information serves to orient the RNA polymerase with respect to the promoter such that the direction of transcription along the template can be anticipated.

RNAP mutants blocked in other functions

To identify mutations that might affect other functions of the RNAP (catalysis, elongation, termination, etc.) we constructed 35 linker insertion mutants of T7 RNAP in which a 6 bp linker (two amino acids) was placed at various positions in the RNAP gene (Gross et al., 1993). These mutants were subjected to a variety of biochemical assays designed to detect blocks in key steps in the transcription cycle. A number of mutants with interesting biochemical phenotypes were identified, some of which are described below.

An additional region involved in promoter recognition

Among the linker insertion mutants were a class of RNAPs that retain nonspecific catalytic activity (i.e., they are able to synthesize poly rG on a poly dC template) but which have lost promoter-binding ability. Some of these mutations map near residue 748, as expected from the above discussion. However, other mutations map closer to the amino terminus of the protein. Two mutants in particular (*ins*144 and *ins*159, which consist of insertions within or before codons 144 and 159) are of particular interest because they lie near a region of T7 RNAP that exhibits significant sequence homology to region 2.4 of the bacterial sigma factor (Gross et al., 1993). This region of sigma factor is known to interact with base pairs in the −10 region of the *Escherichia coli* consensus promoter sequence (Helman and Chamberlin, 1988; Daniels et al., 1990; Siegele et al., 1989; Waldburger et al., 1990). In the crystal structure of T7 RNAP, this region is found within the DNA binding cleft, not far from the region defined by residue 748 (Fig. 3). Together, these two elements of the DNA binding cleft come in contact with the upstream region of the phage promoter, thus defining a sequence specific recognition element. The homology of this region to sigma factor suggests that additional common sequence elements may be found between the phage RNAPs and the multisubunit RNAPs.

Active site mutants

Another interesting class of mutants are those that retain promoter-binding activity, but have lost catalytic activity. Two interesting mutants within this class (*ins*640 and *ins*648) exhibit a characteristic defect in their ability to utilize double-stranded DNA templates but not single-stranded templates. For example, both of these enzymes exhibit significant activity on dC or dI-dC templates, but no activity on a dG:dC template (Gross et al., 1993). We reasoned that the defect in these enzymes might lie in their inability to melt open the double stranded helix, or failure to maintain an association with the template strand during elongation. This was confirmed by the use of synthetic promoters in which the promoter was "premelted" by virtue of the fact that the nontemplate strand in the initiation region was missing; whereas the wild-type enzyme is capable of initiating transcription from a fully duplex promoter as well as the premelted promoter, the two mutant enzymes were capable only of initiation from the premelted promoter (Gross et al., 1993).

The interpretation of these results with regard to the structure of T7 RNAP relied upon a potential similarity between the phage RNAPs and other nucleotide polymerases that was first observed by Delarue et al. (Delarue et al., 1990). These authors noted a homology in three sequence motifs (A–C) found in many nucleotide polymerases, including DNA polymerase and the single subunit DNA-dependent RNAPs. Two of these motifs (A and C) are also found in RNA-dependent RNA polymerases as well RNA-dependent DNA polymerases. In the structure of the Klenow fragment of *E. coli* DNA polymerase I (KF) these

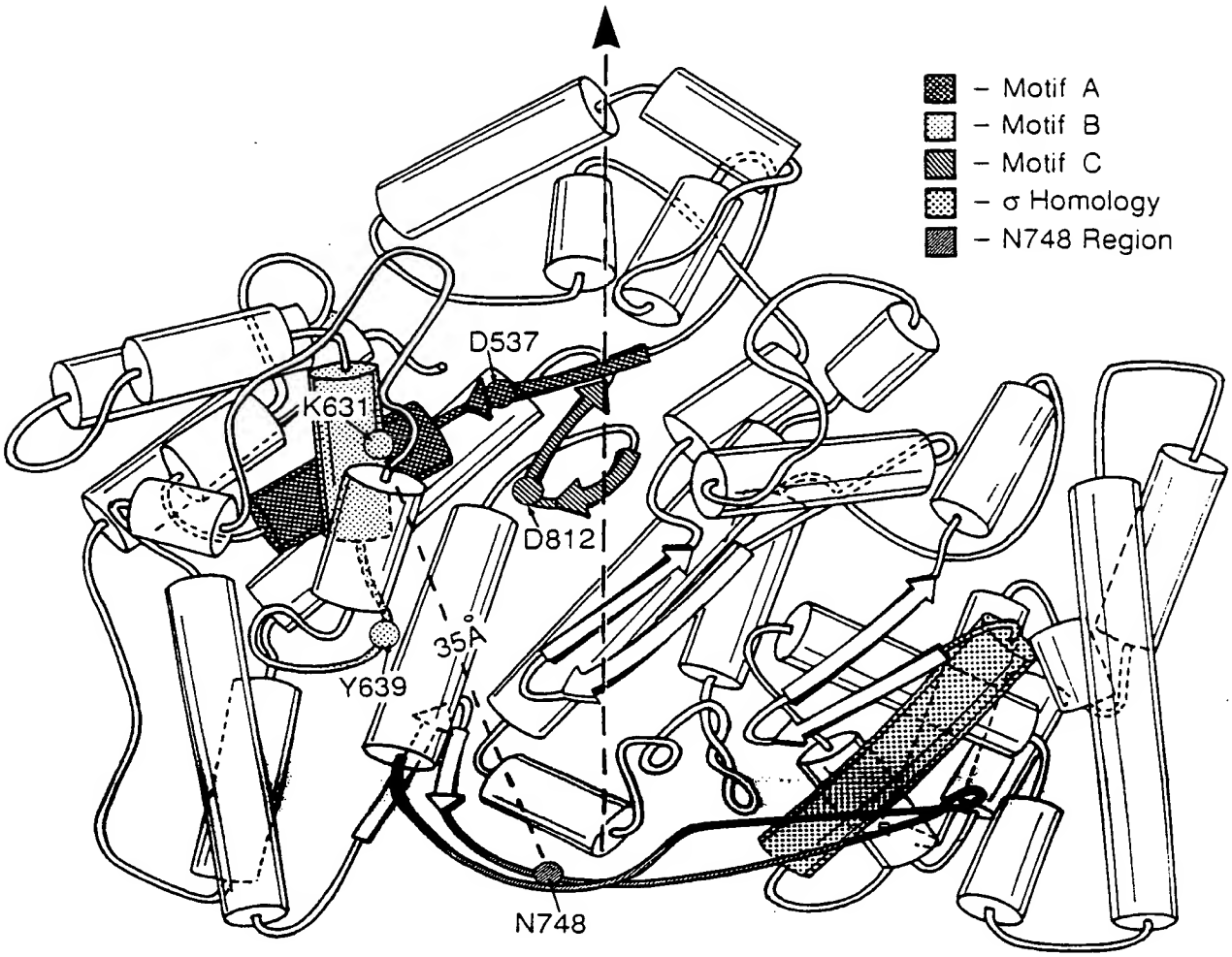

Fig. 3. Structure of T7 RNA polymerase. The schematic depicts T7 RNA polymerase looking into the DNA binding cleft: the axis of the cleft runs vertically, as indicated by the dashed arrow (adapted from Sousa et al., 1993). Structural motifs that are common to other nucleotide polymerases and which define the active site are indicated by selective shading (motifs A, B, and C), as is the region that exhibits homology with sigma factor region 2.4. Key catalytic residues are indicated. Residue N748, which is involved in contacts with the nontemplate bases at −10 and −11, lies on an extended loop at the base of the cleft. The distance from this residue to K631, which may be crosslinked to the initiating nucleotide, is 35 Å (approximately one turn of the double helix).

three regions are located near the active site (Ollis et al., 1985). This finding, and the observation that motif B differed in enzymes that utilize RNA vs. DNA as a template, led Delarue et al. to speculate that these polymerases may have evolved from a common precursor (or may use similar structural motifs to carry out common catalytic functions), and that motif B is likely to be involved in association with the template strand. The two mutations of interest in T7 RNAP (*ins*640 and *ins*648) lie within motif B. The inability of these mutant enzymes to melt open promoters or to remain stably associated with the template strand following initiation is consistent with the proposal that motif B is in association with the template strand.

Certain residues within motifs A, B, and C are highly conserved among all of the polymerases; these include, in particular, K631 in T7 RNAP, which lies in motif B. We and others have shown that this residue may be crosslinked to analogs of the initiating triphosphate, and that the crosslinked analog may subsequently serve as an acceptor in the formation of a phosphodiester bond with the next (incoming) nucleotide in a template-directed manner (Schaffner et al., 1987; Maksimova et al., 1991). Residue K631

must, therefore, be near the acceptor site in the initiation complex, consistent with its proximity to the template strand in the model described above.

More recent crystallographic data at higher resolution show a close structural correspondence between T7 RNA and KF, especially in the regions now referred to as the "polymerase-fold" (Sousa et al., 1993). A similar structural correspondence has been noted for the HIV reverse transcriptase, lending further support to the notion of a common catalytic mechanism for these enzymes (Kohlstaedt et al., 1992).

DISCUSSION

The convergence of genetic and biochemical approaches, as well as the availability of a high resolution crystal structure for T7 RNAP, make this a particularly exciting time to study the structure and function of an RNA polymerase. As a result of this and other work, considerable information is now available concerning the regions of the RNAP that are involved in promoter recognition, transcript elongation, and termination (for recent review, see (McAllister and Raskin, 1993). There is a growing body of evidence that supports the existence of a common polymerase fold among the simple nucleotide polymerases. This fold is likely to comprise the active site required for basic catalytic functions, and to contain elements that are involved in template binding and positioning of the active site. Other functions that are unique to the particular type of polymerase (e.g., promoter recognition and binding for the RNA polymerases, proofreading, and exonuclease functions for the DNA polymerases) are likely to be located elsewhere in the polymerase, possibly in auxiliary domains (see, for example, Fig. 3, in which the promoter recognition site is spatially quite separate from the putative active site).

What about the multisubunit RNA polymerases, do they also share homologies, or have they evolved along a different pathway? It is possible that as a result of the need to maximize the opportunity for regulation, multisubunit enzymes have distributed their corresponding functional motifs among multiple subunits. Sequence alignment programs may be unable to detect highly divergent motifs that are distributed among many protein subunits. A more fruitful approach may involve searching individual subunits for conserved motifs found in the phage-like RNA polymerases. The potential alignment between sigma factor and T7 RNAP suggests that this may prove to be an attractive method of analysis, although a functional role for this region of the phage RNAP must be confirmed. In any event, it is clear that studies of the structure and function of an elegantly simple RNAP like T7 will provide important clues for understanding the functioning of other polymerases.

Acknowledgements — This work was supported by NIH grant GM38147. I am grateful to the members of my laboratory for their performance of these studies, and to Rui Sousa for communicating results prior to publication.

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entirely at discrete DNA sequences, termed promoters and terminators. These define units of transcription, and transcription on such templates is restricted to these regions and is therefore selective (*14*). Both promoter and terminator sites can vary considerably in their structure and in the efficiency and specificity of interaction with RNA polymerase. Thus the rate and efficiency of interaction of RNA polymerase with these loci is of major importance in determining which genetic sequences are expressed in the cell, and at what rate.

There have been extensive studies of the individual steps of the transcription cycle, especially those involved in promoter binding and chain termination. A discussion of the mechanism and specificity of these reactions is not possible in the space available here. The reader is referred to reviews and monographs on promoter structure and binding (*15, 23–26, 29, 30*), RNA chain initiation (*22, 23*), RNA chain elongation (*33, 35, 79*), and RNA chain termination (*27–29, 31, 155*).

With single-stranded DNA templates there is little or no specificity in the sites used for RNA chain initiation, and RNA chain termination occurs randomly throughout the reaction (*156*) to give rather short RNA chains bonded to the template in a DNA–RNA hybrid (*157, 158*). The process may well be brought about by formation of the hybrid structure, leading to premature chain termination, since a very similar reaction occurs with poly(dG)·poly(dC) template when poly(rG) is synthesized (*90*). The product of this reaction is a poly(rG)·poly(dC) hybrid with displacement of the poly(dG) strand (*159*). The random initiation-termination transcription process on single-stranded M13 DNA can be suppressed by *E. coli* single-strand binding protein, leading to selective initiation at a single promoter site (*160*), however there have been relatively few studies of *in vitro* transcription with well-defined single-stranded templates.

155. Yanofsky, C. (1981). *Nature (London)* 289, 751.
156. Maitra, U., Nakada, Y., and Hurwitz, J. (1967). *JBC* 242, 4908.
157. Chamberlin, M., and Berg, P. (1964). *JMB* 8, 297.
158. Sinsheimer, R., and Lawrence, M. (1964). *JMB* 8, 289.
159. Chamberlin, M. (1965). *FP* 24, 1446.
160. Schaller, H., Uhlmann, A., and Geider, K. (1976). *PNAS* 73, 49.

4

Bacteriophage DNA-Dependent RNA Polymerases

M. CHAMBERLIN • T. RYAN

I. Introduction

Infection of a bacterial cell by a bacteriophage leads to a progressive reprogramming of the biosynthetic capabilities of the cell toward synthesis of bacteriophage components. An early and fundamental step is the establishment of a regulated transcription program for the bacteriophage genome. Most bacteriophages—with notable exceptions—depend on the host transcriptional machinery for transcription of genes used early in infection. Late bacteriophage transcription, however, can employ either the host RNA polymerase or an independently synthesized RNA polymerase coded for by the bacteriophage genome. This review deals entirely with the latter enzymes. Other related reviews cover phage trans-

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cription involving bacterial RNA polymerases that have been reprogrammed to read phage-specific transcriptional units (*1–3*), and the genetics and physiology of T7 and the T7-like bacteriophages, including genetics of the T7-like RNA polymerases and their role in bacteriophage growth and development (*4, 5*).

Viral-coded DNA-dependent RNA polymerases were first identified in mammalian viruses as activities carried in the viral particle (*6, 7*). Subsequently, an RNA polymerase coded for by T7 bacteriophage and specific for T7 DNA as template (*8*) was isolated from extracts of infected *E. coli*. Similar enzymes are induced after infection by a variety of T7-like bacteriophages, and are characterized by having only a single polypeptide chain and by being highly specific for the homologous phage DNA template (*4, 9–12*).

Two other kinds of bacteriophage-specified DNA-dependent RNA polymerases are known. Both are under the control of bacteriophages that can induce active transcription in bacterial cells even in the presence of rifampicin, which blocks transcription by bacterial RNA polymerase. An RNA polymerase activity specified by one of these phages, N4, is carried in the bacteriophage particle, and this enzyme, rather than the *E. coli* host polymerase, is responsible for transcription of N4 DNA immediately after infection (*13, 14*). It is a very large protein, consisting apparently of only a single polypeptide chain of molecular weight about 350,000 (*15*). The structure of another RNA polymerase, induced by bacteriophage PBS2, which grows in *Bacillus subtilis*, is more complex. The enzyme seems to contain at least five phage-

1. Losick, R., and Pero, J. (1976). *In* "RNA Polymerase" (R. Losick, and M. Chamberlin, eds.), p. 227. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
2. Losick, R., and Pero, J. (1981). *Cell* 25, 582.
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5. Kruger, D., and Schroeder, C. (1981). *Microbiol. Rev.* 45, 9.
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7. Baltimore, D., Huang, A., and Stamfer, M. (1970). *PNAS* 66, 572.
8. Chamberlin, M., McGrath, J., and Waskell, L. (1970). *Nature (London)* 228, 227.
9. Dunn, J., Bautz, F., and Bautz, E. (1971). *Nature New Biol.* 230, 94.
10. Towle, H., Jolly, J., and Boezi, J. (1975). *JBC* 250, 1723.
11. Korsten, K., Tomkiewicz, C., and Hausmann, R. (1979). *J. Gen. Virol.* 43, 57.
12. Butler, E. (1978). Ph.D. Thesis, University of California, Berkeley, California.
13. Rothman, Denes, L., and Schito, G. (1974). *Virology* 60, 65.
14. Falco, S., Vanderlaan, K., and Rothman-Denes, L. (1977). *PNAS* 74, 520.
15. Falco, S., Zehring, W., and Rothman-Denes, L. (1980). *JBC* 255, 4339.

contain at least five phage-coded components (*16, 17*), and it does not seem to be involved in the early phase of phage transcription as originally thought. Its role in the physiology of PBS2 infection is still not well understood.

This review is divided into sections concerning the T7-like RNA polymerases, about which there is extensive information, and the RNA polymerases from other phages, about which there is somewhat less information.

II. T7-Like Bacteriophages

A. Molecular Properties

1. *Purification*

A number of bacteriophages specify DNA-dependent RNA polymerases similar to the T7 enzyme; these are all morphologically similar to T7 and often show genetic homology as well (*4, 11, 18*). The T7 RNA polymerase is the most thoroughly studied and will be taken as representative of the general class, although individual phage polymerases may show significant differences.

A variety of procedures has been used to purify T7 RNA polymerase, however there is no really satisfactory method that gives high yields of homogeneous and active polymerase. This is primarily due to several factors. The enzyme is rather unstable and loses activity during purification, especially when protein concentrations are low. In addition, there is not a large amount of enzyme induced in infected cells under conditions of wild-type infection.

The original method of Chamberlin *et al.* (*8*) for purification of T7 polymerase employed streptomycin sulfate precipitation to remove nucleic acids, precipitation and extraction with ammonium sulfate, followed by column chromatography on DEAE-cellulose and phosphocellulose. The peak fractions from phosphocellulose were over 90% T7 polymerase protein as judged by SDS gel analysis (*8*). However, the yields and specific activities obtained by this method are relatively low and there is often significant variation in the early steps. Niles *et al.* (*19*) introduced a

16. Clark, S., Losick, R., and Pero, J. (1974). *Nature (London)* 252, 21
17. Clark, S. (1978). *J. Virol.* 25, 224.
18. Hyman, R., Brunovskis, I., and Summers, W. (1974). *Virology* 57, 189.
19. Niles, E., Conlon, S., and Summers, W. (1974). *Biochemistry* 13, 3904.

modified procedure in which nucleic acids and T7 polymerase are precipitated from the extract with polyethyleneimine, and the T7 polymerase is then extracted from the precipitate with salt. These fractions are then fractionated with ammonium sulfate, and then by column chromatography on phosphocellulose, DEAE-cellulose, and hydroxylapatite, respectively. This procedure gives good yields of enzyme activity through the phosphocellulose step and is quite reproducible (*20*). These fractions are only of moderate specific activity and contain contaminating peptides, but they give normal amounts of the large T7 transcripts, as measured by RNA gel analysis of the products, and are quite adequate for transcriptional analysis (*19, 20*) or preparation of specific labeled RNAs. Such fractions have been kept at −20° in 50% glycerol solutions without substantial loss in activity for many years (*20*). Further purification of these fractions by heparin agarose chromatography gives enzyme of very high specific activity (*20*), but these fractions have been somewhat less stable, possibly due to the low protein concentrations involved.

An alternative modification of the Niles *et al.* (*19*) procedure was reported to give homogeneous T7 polymerase (*21*), although no yields or specific activities were described. However, subsequent studies indicate that these fractions may be contaminated with a single-strand-specific endonuclease, and gel analysis suggests that as much as 30–50% of the protein can be in peptides other than T7 polymerase (*22*). Chromatography of these fractions on T7 DNA cellulose may give homogeneous enzyme (*22*), but no yields are reported and the capacity of the column is said to be quite low.

Similar procedures are generally applicable for the purification of T3 RNA polymerase. Bailey and McAllister (*23*) have isolated the T3 RNA polymerase using the polyethyleneimine procedure through the phosphocellulose step, followed by chromatography on heparin-agarose and phosphocellulose.

An alternative procedure for purification of T3 RNA polymerase (*24*) takes advantage of the fact that the enzyme in cell extracts is easily sedimented with cell debris, probably due to binding to ribosomes (*9*). It can be eluted from the pellet with salt solutions and subsequently fractionated by column chromatography. The procedure is reported to give reasonable recoveries (~20%) of enzyme of good specific activity

20. Kassavetis, G., and Chamberlin, M. (1979). *J. Virol.* 29, 196.
21. Oakley, J., Pascale, J., and Coleman, J. (1975). *Biochemistry* 14, 4684.
22. Strothkamp, R., Oakley, J., and Coleman, J. (1980). *Biochemistry* 19, 1074.
23. Bailey, J., and McAllister, W. (1980). *Nucleic Acids Res.* 8, 5071.
24. Chakraborty, P., Sarkar, P., Huang, H., and Maitra, U. (1973). *JBC* 248, 6637.

(~600,000 units/mg), which gives a single band on SDS-polyacrylamide gels.

Isolation of the RNA polymerase from *Salmonella typhimurium* infected with phage SP6 has proved to be somewhat easier than for the T7 enzyme, due in part to the greater stability of SP6 enzyme (*12, 25*). After removal of nucleic acids with streptomycin and ammonium sulfate precipitation, the enzyme is chromatographed on phosphocellulose, Blue Dextran-Sepharose, and Bio-Gel P200, respectively, to give a homogeneous protein fraction. This gives yields of up to 30% overall of SP6 RNA polymerase activity and specific activities of 700,000 units/mg (*12, 25*).

2. *Enzyme Assay*

T7 RNA polymerase is usually assayed by following incorporation of radioactively labeled nucleotide into acid-insoluble material in the presence of T7 DNA as template. The reaction shows an absolute requirement for the four ribonucleoside triphosphates, Mg^{2+}, and T7 DNA (*8, 26*). T7 DNA can be replaced by other duplex DNA templates that bear specific T7 polymerase promoter sites (see below) or by synthetic polynucleotides such as $[dG)_n \cdot (dC)_n$, $(dI)_n \cdot (dC)_n$, or poly(dC). The rate of synthesis is optimal between pH 7.7 and 8.3. The rate of synthesis is highly sensitive to reaction temperature (*26*) and falls off rapidly below 37°; there is about a twofold reduction at 30°. This may be due, in part, to a requirement for DNA strand separation in a rate-controlling step (*27*), but the rate falls off nearly as rapidly with single-stranded $poly(dC)_n$ as template (*26*), suggesting that other steps or temperature-dependent changes in enzyme conformation are also involved.

The rate, and also the extent, of T7 RNA synthesis is affected by sulfhydryl reactive agents, such as *p*-chloromercuribenzoate; hence a thiol, such as dithiothreitol or β-mercaptoethanol, is included in the reaction solution. Similarly, the reaction, especially with early enzyme fractions, shows an enhancement by, or even complete dependence on, the addition of bovine serum albumin (*8*). This may be due to the high sensitivity of the T7 polymerase to inhibition by polyanionic compounds (*28*).

Under optimal conditions the reaction continues at constant rate for about 20–30 min after a short (10–15 sec) lag. However, this extended period of synthesis involves many cycles of transcription initiation, elongation, and termination for each active polymerase. The longest of the

25. Butler, E., and Chamberlin, M. (1982). *JBC*, in press.
26. Chamberlin, M., and Ring, J. (1973). *JBC* 248, 2235.
27. Oakley, J., Strothkamp, R., Sarris, R., and Coleman, J. (1979). *Biochemistry* 18, 528.
28. Chamberlin, M., and Ring, J. (1973). *JBC* 248, 2245.

transcription units controlled by a strong (class III) T7 promoter is 12,000 bases (29), corresponding to a transit time for T7 RNA polymerase of 60 sec at 200 nucleotides per sec (*30*), and the average transit time for class III transcriptional units is 20–25 sec. Hence each active T7 RNA polymerase must repeat the transcription cycle about 3 times each min.

A consequence of this extensive recycling during transcription with T7 DNA is that the reproducibility of assays falls off rapidly at times over 5–10 min (J. Ring and M. Chamberlin, unpublished studies). This is probably because T7 RNA polymerase is not highly stable at 37°, and hence small variations in reaction conditions, glassware, etc., affect the lifetime of enzyme released during the recycling process. Since loss of a small fraction of enzyme is multiplied exponentially in recycling, there is a disproportionate effect on the extent of incorporation at longer reaction times. For example, the presence of a factor or condition that slightly destabilizes free T7 RNA polymerase, so that 5% of the free enzyme is inactivated prior to each round of chain initiation, will reduce incorporation in a 10-min assay to $100 \times (0.95)^{30} = 20\%$, since there are about 30 rounds of transcription involved. Because of this, reaction times of no more than 5–10 min should be used.

The definition of a unit of T7 RNA polymerase activity has generally been based on measurement of the rate of reaction in a 10-min incubation under specified reaction conditions with T7 DNA as template (*8*, *28*). One unit is the amount of enzyme needed to give a rate of incorporation of 1 nmol of labeled substrate per hour under these conditions.

Since T7 RNA polymerase elongates RNA chains at ~200 nucleotides per sec at 37° (*30*), it can be calculated that a homogeneous protein of MW 100,000 involved solely in chain elongation at this rate would have a specific activity of $\sim 1.8 \times 10^6$ units/mg. However, T7 RNA polymerase requires ~10–15 sec to initiate an RNA chain (*26*), and each active polymerase does so an average of three times per minute. Therefore, the maximum specific activity of fully active T7 RNA polymerase in these assays is probably about 1×10^6 units/mg. This neglects any time required for release of enzyme at RNA chain termination, and assumes no inactivation of enzyme during the recycling reaction. This maximum value for the specific activity should be compared to the highest specific activities reported of ~600,000.

From the above considerations it is clear that the normal assay for T7 RNA polymerase, although useful for following the presence of active RNA polymerase during fractionation, does not give a quantitative assay

29. Carter, A., Morris, C., and McAllister, W. (1981). *J. Virol.* 37, 636.
30. Golomb, M., and Chamberlin, M. (1974). *JBC* 249, 2858.

for the molar concentration of active RNA polymerase present. Changes in the activity do not necessarily reflect possible changes in the specificity of the enzyme, or in the rate or efficiency with which it carries out individual steps in the transcription cycle. This is even more true when reaction conditions are altered, inhibitors are present, etc., where it cannot be assumed that the rates or efficiencies of the different steps of the transcription cycle are equally affected.

It would be highly desirable to have a quantitative RNA polymerase assay involving a single transcriptional cycle, similar to that developed for bacterial RNA polymerases (*31*). The requirements for such an assay are set forth elsewhere in this volume (*32*). It is clear that such an assay for T7 RNA polymerase cannot be devised with T7 phage DNA. It contains far too many transcription units that vary in transit time from about 3 to 60 sec. Since chain initiation, or establishment of a normal rate of chain elongation, requires about 10–15 sec, it is clearly impossible to separate chain elongation from initiation and termination. Such a separation should be possible with cloned T7 polymerase promoters inserted in large cloning vectors. In principle, if there were no chain termination, and transcription were initiated on an intact circular DNA, RNA chain elongation would be continued more or less indefinitely and a quantitative measure of the concentration of active RNA polymerase could be obtained simply from the rate of incorporation and the elongation rate.

Unfortunately, although cloned phage polymerase promoters are available (*33*), available vectors contain several partially effective terminator sites [Ref. (*33*), and D. Roulland, unpublished observations]. Hence, although these plasmid DNAs can generate extremely large amounts of RNA, *in vitro*, there is still the same problem of separating the different reaction steps. In view of the importance of obtaining a quantitative T7 RNA polymerase assay, it would be useful to attempt to develop better DNA templates for these assays by systematically attempting to remove *in vitro* termination sequences from the cloning vectors.

The biochemical properties and assay procedures for other T7-like phage RNA polymerases are generally similar to those of the T7 polymerase, and have been studied for the T3 RNA polymerase (*24*, *33–37*), *Pseudomonas* phage gh-1 (*10*), and *Salmonella* phage SP6 (*12*, *25*).

31. Chamberlin, M., Nierman, W., Wiggs, J., and Neff, N. (1979). *JBC* 254, 10061.
32. Chamberlin, M. (1981). Chapter 3, this volume.
33. McAllister, W., Morris, C., Rosenberg, A., and Studier, F. (1981). *JMB*, 153, 527.
34. Dunn, J., McAllister, W., and Bautz, E. (1972). *EJB* 29, 500.
35. McAllister, W., Kupper, H., and Bautz, E. (1973). *EJB* 34, 489.
36. Salvo, R., Chakraborty, P., and Maitra, W. (1973). *JBC* 248, 6647.

These different enzymes all show a requirement for their own specific class of promoter sites and generally will not use heterologous templates (see Section II.B). However all of the T7-like RNA polymerases studied thus far will use the synthetic polynucleotides $(dG)_n \cdot (dC)_n$ or poly(dC) as templates. These templates lack specific promoter sequences: transcription on such templates probably reflects simply the general catalytic activity of the polymerase in a reaction where specific promoter binding has been bypassed.

3. *Physical and Chemical Properties*

Highly purified T7 RNA polymerase preparations contain a single polypeptide chain that is the protein product of T7 gene 1 (*8*). The mobility of the protein on SDS-polyacrylamide gels corresponds to a MW of 107,000–110,000 (*8*, *19*). No other factors or components appear to be required for enzymatic activity. The active RNA polymerase has a sedimentation coefficient of 5.9 to 6.3 S (*8*, *19*), which is consistent with its existing as a monomer of MW ~100,000. From these observations it is concluded that the active form of T7 RNA polymerase consists of a single subunit of MW ~100,000 (*8*).

The T7 gene coding for T7 polymerase has been cloned and sequenced, giving both the amino acid sequence and size of the T7 polymerase protein (*38*). T7 polymerase protein contains 883 amino acid residues, corresponding to a MW of 98,092. The amino acid composition is given in Table I.

The true molecular weight is significantly lower than that estimated by SDS-polyacrylamide gel electrophoresis. This may be due to inaccuracies in the molecular weights of marker polypeptides in this molecular weight range (*32*); however, it is more likely that the T7 polymerase protein displays an abnormal mobility in SDS-polyacrylamide gels due to some feature of its structure. The sigma subunit of *E. coli* RNA polymerase displays a very abnormal mobility and gives an apparent MW of 80,000–90,000 (*39*), although the true MW is close to 70,000 (*40*).

Physical studies of T7 RNA polymerase have been handicapped by lack of availability of large amounts of homogeneous, fully active, protein (see Section II.A.1). Several studies of physical and chemical properties have been carried out on purified T7 RNA polymerase preparations, however the preparations are likely to have contained significant amounts of contaminating proteins, as well as unknown amounts of enzymatically inactive T7 RNA polymerase, and physical and chemical parameters that would be affected by this contamination are potentially in error. The amino acid composition of one such preparation does not agree well with that determined by DNA sequencing; this is especially true for the amino acids Tyr and Trp (*19*). Values of $\bar{v}$ and $E_{1\%}^{280}$ calculated in these earlier studies (*19*) should probably be recalculated using the amino acid composition determined from the DNA sequence (*38*). Analysis of preparations of T3 RNA polymerase (*41*) and SP6 RNA polymerase (*12*, *25*) using SDS-gel electrophoresis shows that these proteins have mobilities slightly greater than that of the T7 enzyme. Hence the true molecular weights of these enzymes are probably slightly lower than those of the T7 enzyme, assuming that the mobilities of these proteins resemble the T7 polymerase.

37. Chakraborty, P., Bandyopadhyay, P., Huang, H., and Maitra, U. (1974) *JBC* **249**, 6901.
38. Stahl, S., and Zinn, K. (1981). *JMB* **148**, 481.
39. Lowe, P., Hager, D., and Burgess, R. R. (1979). *Biochemistry* **18**, 1344.
40. Burton, Z., Burgess, R., Lin, J., Moore, D., Holder, S., and Gross, C. (1981). *Nucleic Acids Res.* **9**, 2889.

TABLE I

AMINO ACID COMPOSITION OF T7 RNA POLYMERASE[a]

Amino acid	Predicted
Alanine	100
Arginine	40
Asparagine	37
Aspartic acid	41
Cysteine	13
Glutamine	32
Glutamic acid	65
Glycine	55
Histidine	21
Isoleucine	52
Leucine	71
Lysine	63
Methionine	24
Phenylalanine	34
Proline	37
Serine	48
Threonine	49
Tryptophan	18
Tyrosine	23
Valine	60
Total	883
Molecular weight	98,092

[a] Data are from Ref. (*38*).

41. Beier, H., and Hausmann, R. (1974). *Nature (London)* **251**, 538.

T7 RNA polymerase contains tightly bound Zn^{2+}, which appears to be required for catalytic activity (*42*). Other template-dependent nucleotidyl-transferases also appear to contain Zn^{2+} (*43*). It has been reported that addition of Zn^{2+} to some preparations of T7 RNA polymerase enhances T7 RNA polymerase activity (*42*).

The phage RNA polymerases coded by T3 and SP6 phages are cleaved by trypsin to give smaller subfragments that are catalytically active (*25, 44*). In the case of the SP6 enzyme, the activity with SP6 DNA, which depends on specific promoter sites, is lost much more rapidly than the activity with $(dI)_n \cdot (dC)_n$, which does not depend on such sites (*25*). Therefore, it appears that a much smaller subfragment of the phage RNA polymerase can carry out the catalytic functions of the enzyme.

What parts of the phage RNA polymerase protein are involved in the biochemical reactions that specify each of the steps in synthesis of an RNA chain? In the case of the bacterial RNA polymerases, at least four kinds of subunits are involved, whereas for the phage enzyme only a single polypeptide is needed. This means that there must be multiple active sites and catalytic domains on the phage polymerase molecule.

Although the bacterial RNA polymerases are more complex molecules, for functional studies this is an advantage of sorts since the subunits can be distinguished and the role of each one in the reaction probed separately. For the phage enzyme a more limited number of approaches is possible.

Ideally one would like to have the three-dimensional structure of the phage RNA polymerase molecule, together with structures for the enzyme bound to a promoter site, and perhaps with substrates. This goal is certainly feasible in terms of current X-ray crystallographic techniques, but will depend on the development of procedures for isolation of large amounts of a homogeneous and fully active phage RNA polymerase. This has proved to be a difficult goal.

An alternative would be to isolate mutant RNA polymerases and study their properties, possibly in conjunction with mutant promoter sites. Unfortunately, this classical approach has not been particularly useful in studying the phage RNA polymerase molecule.

Another approach to probing the structural basis of promoter selectively for the phage RNA polymerases was initiated by Beier and Hausmann (*41*). They took advantage of the fact that viable, intergenic hybrids could be made between T7 and T3, and constructed a series of recombinant phage strains that contained hybrid gene 1 regions, using various pairs of T7 and T3 gene 1 *amber* mutants. The use of such RNA polymerase variants to investigate the RNA polymerase promoter interaction was made possible by the fact that the two parental gene 1 products differ significantly in promoter specificity (see Section II,B,1).

When the RNA polymerases of progeny from these crosses were tested for their ability to transcribe both phage templates, a range of template specificities was observed (*41, 45*). Each hybrid enzyme, in addition to having a preference for either T7 or T3 DNA, was found to be capable of transcribing the heterologous template to some extent. Since the map positions of the T7 and T3 *amber* mutations used in the construction of these hybrids were known, it was possible to predict the positions of the crossover events, and, in turn, correlate the template preference (i.e., promoter specificity) exhibited for these enzymes with the presence of a particular region of the gene 1 sequence. The region in question was identified as being between 0.7 and 0.78 gene 1 length, a distance corresponding to approximately 75 amino acids.

However, recent studies on these hybrid gene 1 sequences have indicated a somewhat more complex situation concerning the functional anatomy of the enzyme (*46*). Using restriction sites to map the recombination events within the gene 1 region, it was discovered that in all 8 cases examined the genetic constitution of the hybrid gene 1 region differs significantly from that predicted based on the positions of the *amber* mutations in the parental phage. More specifically, this analysis revealed that the active hybrid gene 1 sequences were often the result of complex combinations of genetic rearrangements, including multiple crossovers, and presumably reversions and/or secondary mutations. This suggests that active hybrid T7/T3 gene 1 sequences are rarely formed by single genetic rearrangements, and that promoter selectivity is likely to be a function of more than one region of the polypeptide chain of the enzyme.

It now appears that the region from approximately 23 to 50%, together with the carboxyl end of the molecule, are important in promoter recognition (*46*). A perfect correspondence was found for the level of heterologous activity with the origin of the DNA sequences between 23 and 50%.

In conclusion, it no longer seems feasible to define a single small region as coding for a discrete functional domain in RNA polymerase that uniquely specifies template selectivity. At least two domains on the protein, separated from one another on the polypeptide chain, are involved.

42. Coleman, J. (1974). *BBRC* **60**, 641.
43. Mildvan, A., and Loeb, L. (1979). *CRC Crit. Rev.* **6**, 219.
44. Bautz, E. (1976). *In* "RNA Polymerase (R. Losick and M. Chamberlin, eds.), p. 273. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
45. Hausmann, R., and Tomkiewicz, C. (1976). *In* "RNA Polymerase (R. Losick and M. Chamberlin, eds.), p. 731. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
46. Ryan, T., and McConnell, D. J. (1982). *J. Virol.*, in press.

It is not yet clear whether these two functional domains form a single active site, which in turn interacts with the promoter. The observed inadvertant selection of extra crossovers, in addition to those originally selected for, might support this idea. Alternatively, it is conceivable that the phage enzyme might behave in a somewhat analogous manner to that found for the *E. coli* enzyme, where enzyme promoter interaction seems to involve two separate specific interactions between the enzyme complex and the promoter sequence (*47, 48*). Evidence that there may be two separable DNA sequences involved in the recognition process at the phage RNA polymerase promoter is discussed in the next section.

B. Catalytic Properties

1. *Transcriptional Maps and Template Specificity*

T7 RNA polymerase and related polymerases carry out DNA-directed synthesis of RNA from nucleoside triphosphate substrates. Synthesis of poly(rA) in a reaction dependent on single-stranded DNA and elevated substrate concentrations has also been reported (*36*).

The template specificity of the T7-like phage RNA polymerases is quite striking. Unlike the bacterial RNA polymerases, the phage enzymes normally use only their homologous DNA templates at a substantial rate (*8–10, 24*). This suggested originally that the phage RNA polymerases were highly specific for particular promoter sites (*8, 9, 26*) this was confirmed originally by the mapping of these sites on the T7 and T3 genomes by the transcription of nuclease-digested templates (*49, 50*), of hybrid phage DNAs (*51*), and by *in vitro* translation (*52*). The identity, sequence, and position of what are probably all of the promoters for T7 RNA polymerase on the T7 genome has been determined by cloning and sequencing the regions involved (*33, 53*). When the entire nucleotide sequence of T7 phage is known, it should be possible to write the exact transcriptional map for the entire phage genome.

The resulting transcriptional maps of the T7 and T3 genomes are very similar, although the two polymerases are quite different in their transcriptional specificity (*9*). All of the transcripts initiated by T7 RNA

47. Siebenlist, U., Simpson, R., and Gilbert, W. (1980). *Cell* **20**, 269.
48. Rosenberg, M., and Court, M. (1979). *Annu. Rev. Genet.* **13**, 319.
49. Golomb, M., and Chamberlin, M. (1974). *PNAS* **71**, 760.
50. Golomb, M., and Chamberlin, M. (1977). *J. Virol.* **21**, 743.
51. Beier, H., Golomb, M., and Chamberlin, M. (1977). *J. Virol.* **21**, 753.
52. Sites, E. and Condit, R. (1975). *JMB* **98**, 57.
53. Dunn, J. and Studier, F. (1981). *JMB* **148**, 303.

polymerase on T7 DNA *in vivo* and *in vitro* are read from the r strand of the DNA (*8*), from left to right as the standard genetic and physical map is written. The T7 transcription units are arranged in two overlapping clusters, which share common terminator sites at ~61 and 100%, respectively, on the T7 map (See footnote (*53a*) and Refs. *29, 49, 50, 55*).

There are three classes of T7 polymerase promoters on T7 DNA; class II and class III promoters govern transcription of genes in two different regulatory classes (*33, 49, 56, 57*), while the final class is probably involved in replication initiation. Class III promoters are used at a much greater rate than class II promoters *in vitro* (*49, 55, 58*); this may be related to the fact that genes in class III transcription units continue to be expressed throughout infection, whereas genes in class II transcription units cease to be expressed at late times (*33, 56, 57, 59*).

There are five strong T7 class III promoters located at map positions 46.5, 55, 57.1, 70, and 87 (*29*). An additional class III promoter giving rise to a very large T7 RNA on polyacrylamide gels (T7 species I RNA) had been positioned near 62% (*49–51*); however no promoter has been found in this region (*29*), and it is likely that this RNA band is actually composed of a mixture of large RNA species initiated at class II and III promoter sites that result from readthrough of the 61% terminator (*29*). Another strong promoter originally designated a class III promoter (*49*) is located at 98.3% (T7 species VI RNA) and may play a role as a replication initiation site (*60*).

The strong class III promoters account for over 90% of the *in vitro* transcripts by T7 polymerase from T7 DNA under normal conditions (*30*). Transcripts initiated from the first three sites are terminated at a terminator at about 61% (*29, 49, 52*), while transcripts from the other two sites end at a terminator near 100% or run off the end of the DNA (*29, 49, 55*).

The class II promoters are much weaker *in vitro* and are located between 14.6 and 44.4% on the T7 genome (*29*). Thirteen class II promoters have been identified; these all give transcripts that read into the T7 polymerase 61% terminator (*29*). Because these are weak promoters that

53a. The standard T7 physical map is measured from 0 (left end) to 100% (right end) and contains 40,000 bp (*54*). Positions are noted as % T7 unless stated otherwise.
54. Studier, F., Rosenberg, A., Simon, M., and Dunn, J. (1979). *JMB* **135**, 917.
55. Kassavetis, G., and Chamberlin, M. (1979). *J. Virol.* **29**, 196.
56. Studier, F. W. (1972). *Science* **176**, 367.
57. McAllister, W., and Barrett, C. (1977). *Virology* **82**, 275.
58. McAllister, W., and Carter, A. (1980). *Nucleic Acids Res.* **8**, 4821.
59. McAllister, W., and Wu, H. (1978). *PNAS* **75**, 804.
60. Studier, F., and Rosenberg, A. (1981). *JMB* **153**, 503.

give rather large transcripts, and because of the low resolution of early RNA gel systems, these promoters were not originally identified in the products from intact T7 DNA templates (*30, 49*). However transcription of restriction endonuclease fragments and cloned T7 segments, together with enhanced resolution of transcripts on RNA gels, has allowed detection and positioning of the promoters (*29*). This was facilitated by the finding that transcription from class II promoter sites is enhanced selectively at low Mg^{++} concentrations (*58*). Exact positions have been assigned for most of these promoters by DNA sequencing, taking advantage of the characteristic T7 promoter sequence (*53, 61–65*).

The transcriptional map for T3 RNA polymerase on T3 DNA is very similar to that found for T7 (*23, 50, 51*). Here again all transcripts are read from the r-strand of the DNA and there are two classes of promoter sites. Five strong class III promoters form two sets of overlapping transcription units from near 46% to a terminator near 59%, and from 67% to the end (100%). Eleven weaker class II promoters are spread throughout the left half of the genome from 1.5 to 44% and all read to a strong terminator near 59% (*66*).

The sequences of T7 promoter sites show characteristic sequence homologies in the region of the RNA start site. All of the strong class III promoters share a common 23-base sequence (*53, 61, 62*), which begins 17 base-pairs (bp) prior to the nucleotide coding for the 5' terminus of the T7 polymerase transcript (−17), and continues 6 bases past that site (+6). The class II promoters are quite similar (*53, 63–66*), but have slightly altered sequences in this region of homology (see Table II). In analogy with the bacterial RNA polymerases it is likely that these sequences contain the DNA residues involved in recognition of the promoter site. The high degree of sequence identity among the phage RNA polymerase promoters is especially striking in view of the great diversity of bacterial RNA polymerase promoter sequences (*47, 48*). This probably reflects a greater demand for variation of promoter efficiency and interaction with regulatory factors for the bacterial promoter sites.

61. Rosa, M. (1979). *Cell* 16, 815.
62. Rosa, M. (1981). *JMB* 147, 199.
63. Oakley, J., and Coleman, J. (1977). *PNAS* 74, 4266.
64. Pinayotatos, N., and Wells, R. (1979). *Nature (London)* 280, 35.
65. Bouthroyd, J., and Hayward, R. (1979). *Nucleic Acids Res.* 7, 1931.
66. Carter, A., and McAllister, W. (1981). *JMB* 153, 825.
67. Adhya, S., Basu, S., Sarkar, P., and Maitra, U. (1981). *PNAS* 78, 147.
68. Kassavetis, G., Butler, E., Roulland-Dussoix, D., and Chamberlin, M. (1982). *JBC*, in press.

TABLE II

DNA SEQUENCES FOR PROMOTER SITES USED BY T7, T3, AND SP6 PHAGE RNA POLYMERASE[a]

Phage and promoter	Map location	Nucleotide sequence (−15 −10 −5 +1 −5 bp)
T7 class III consensus		T A A T A C G A C T C A C T A T A G G G A G A
T7 class II		
ϕ1.1A	14.6	C - - - - - - - - - - - - - - - - - A - A -
ϕ1.1B	14.8	- - - - - - - - - - - - - - - - - - A G A -
ϕ1.3	15.9	- - - - - - - - - - - - G - - - - - - - C -
ϕ1.5	19.3	- - - - - - - - - - - - - - - - A - - - A G - T
ϕ1.6	19.6	- - - - - - - - - - - - - - - - A - - - A G A C
ϕ2.5	22.6	- - - - - - - - - - - - - - - - - - T A - G A -
ϕ3.8	27.9	- - - T G A - - - - - - - - - A - - - - - - -
ϕ4.3	33.3	- - - - - - - - - - - - - - - - A - - - A G A C
ϕ4.7	34.7	C T - - T - - - - - - - - - - - - - - A G A C
T3	1.2	- - T - T A C C - - - - - - - A - - - - - A T
	22.5	- - - - T A C C - - - - - - - A - - - - - A C
	44.5	A - T - - A C C - - - - - - - A - - - - - A G
	51.5	A - T - - A C C - - - - - - - A - - - - - -
	16.1	- - - - - A C C - - - - - - A G A - - - - - -
SP6 class III		A T T - - G - T G A - - - - - - - - A A T A G

[a] T7 class III sequence is from Rosa (*61, 62*). T7 class II promoter sequences are from the summary by Dunn and Studier (*53*) and from Carter and McAllister (*66*). For sequences other than the consensus class III sequence, bases are shown only if there is a difference between that sequence and the class III sequence. The T3 sequence designated 1.2 is that of Adhya *et al.* (*67*) and is for a T3 promoter near the left end of T3 DNA. Map positions and sequences for this and the other T3 promoters shown are from Ref. (*23*) and from unpublished studies kindly communicated by Dr. W. McAllister. For T3 it has not yet been clearly determined which promoters are class II or class III. The SP6 sequence was determined by E. Butler (unpublished studies) using a *Hind*III—*Bgl*II fragment spanning the region 39,450 bp to 41,100 bp from the left end of SP6 DNA (*12*). This fragment contains a strong SP6 promoter (*68*); the corresponding RNA sequence was determined by M. Gilman (unpublished studies). Map locations are distances from the left end of the DNA molecule, expressed in percentages of the total length. The underlined nucleotide in the first sequence is the transcriptional start site; this is designated +1 by the standard nomenclature (*48*).

The different T7-like RNA polymerases all seem to have evolved their own distinct promoter recognition specificity. T7 RNA polymerase uses T3 polymerase promoter sites on T3 DNA poorly or not at all, (*68a*) while T3 RNA polymerase uses T7 promoter sites on T7 DNA weakly but specifically (*50*). This suggested that there might be a partial homology between T7 and T3 specific promoter sites, and sequencing of a T3 promoter site confirmed this notion [Table II, Ref. (*67*)]. Although there are differences between the T3 promoter sequence and the 23-base sequence of strong T7 class III promoters, there is a region of strong homology stretching from −9 to +4, and the region around −15 also shows strong homology if it is assumed that the GA at −11/−10 on T7 is replaced by the single base, C, in T3 (W. McAllister, personal communication).

Comparative studies of a number of T7-like bacteriophages that grow on different bacterial strains show that these phages all induce phage-coded RNA polymerases of MW about 100,000, and show patterns of growth and regulation similar to T7 (*11*, *12*). However these phages are generally not closely related to T7 or T3 in protein or nucleotide sequences (*11*, *12*). The different phage RNA polymerases all show distinct promoter specificities in that they use their homologous phage DNA as template, but do not use heterologous templates such as T3 or T7 DNA (*10–12*). Since these phages may well have evolved from a common ancestor (*4*, *11*), this suggests that specific promoter sequences can evolve rapidly, along with other portions of the phage genome, despite the presence of about 20 such sites on the genome, which must change in concert with any alteration in the specificity of the polymerase.

A promoter site for the SP6 phage RNA polymerase has been cloned and sequenced (E. Butler, personal communication). The SP6 RNA polymerase does not use T7 or T3 promoter sites and shows no DNA sequence homology by DNA hybridization (*12*, *25*). However the SP6 specific promoter sequence is strikingly similar to the T7 and T3 sequences (Table II) and bears identical sequences from −3 to −7 bp and at the start site! This suggests that, although SP6 appears unrelated to T7 at the level of gross nucleotide sequences, there may be significant homologies at a finer level of analysis. Furthermore, although the T3, T7 and SP6 promoter sites are specific for their particular RNA polymerases, the differences are primarily due to changes in the part of the conserved promoter sequence from −8 to −17 bp.

The result is reminiscent of the situation with promoters for bacterial RNA polymerases, where there are three regions of DNA sequence homology centered about −10 and −35 bp and at the start site that may play quite different roles in the promoter binding and RNA chain initiation process (*47*, *48*).

68a. While the T3 polymerase-specific promoter sites on T3 DNA are not used by T7 polymerase at an appreciable rate, T7 polymerase does use T3 DNA as an effective template (*9*, *24*). This is due to the presence of a strong promoter site for T7 polymerase near 84% on the T3 genome (*49*, *50*), which has a sequence identical to the T7 class III promoter consensus sequence (66). This site is not used at an appreciable rate by T3 RNA polymerase and may be a vestige of the evolution of T3 from a T7-like ancestor. It is possible that T7 polymerase can use the true T3 promoter sites at a low rate, and in fact this is plausible in view of the homology they show with T7 promoter sites. However, the presence of the strong class III-like T7 promoter site on T3 DNA may mask this low rate of use.

69. Rosa, M., and Andrews, N. (1981). *JMB* 147, 41.

2. *Rates and Mechanism of Transcription Cycle Steps*

As in the case of bacterial RNA polymerases, synthesis of a single RNA chain beginning with free T7 RNA polymerase and a template DNA involves a sequential series of steps—the transcription cycle (*32*). These are usually designated as template or promoter binding, RNA chain initiation, RNA chain elongation, and RNA chain termination and release. In the case of the bacterial enzymes, each step involves a complex series of reactions (*32*), and this is likely to be true for the phage polymerases as well (*44*, *70*). However, much less is known about the individual transcription cycle steps carried out by the phage enzymes. This is due primarily to the fact that the phage polymerases do not form highly stable promoter complexes in the absence of substrates, as is found for the bacterial enzyme (*21*, *26*, *28*, *35*, *70–72*). In addition, because of the rapid RNA chain elongation rate (see Section II,A,2) the time needed to complete even a very long transcription unit is usually much less than 60 sec. These two properties of the phage RNA polymerases have made it difficult to separate the individual steps of the transcription cycle for detailed study, as has been possible with the bacterial enzyme.

Despite these difficulties, some features of the individual steps are known. Specific promoter binding by the T3 or T7 RNA polymerases can be demonstrated in the absence of nucleoside triphosphates by the nitrocellulose filter binding procedure (*21*, *71*). Rather large amounts of the enzyme are needed; a 50- to 100-fold molar excess of RNA polymerase protein gives about 50% retention of labeled phage DNA (*21*, *71*). This is probably due to the fact that the binding constant for T7 RNA polymerase to its promoter sites is very low ($\sim 10^7\ M^{-1}$), rather than to a low efficiency of retention of complexes on filters (*26*), since efficient retention of T7 DNA fragments that bear only a single promoter has been observed (*27*).

70. Bautz, E. (1973). *FEBS Lett.* 36, 123.
71. Chakraborty, P., Salvo, R., Majumder, H., and Maitra, U. (1977). *JBC* 252, 6485.
72. Salvo, R., Chakraborty, P. R., and Maitra, U. (1973). *EJB* 32, 645.

The complexes formed with T3 polymerase dissociate quite rapidly ($t_{1/2}$ ~ 60 sec) when unlabeled DNA is added (*71*). Since about 20 promoter sites are involved on each DNA, the intrinsic dissociation rate for an individual polymerase–promoter complex can be no more than a few seconds (*73*). Binding of T7 RNA polymerase at its cognate promoter leads to opening of DNA in the region from −5 to +1, as shown by cleavage of the nontranscribed DNA strand (I strand) with a single-strand-specific endonuclease (*22*). This gives direct evidence that T7 RNA polymerase, like the bacterial enzyme (*47*), directly opens base-pairs at the promoter to form an open promoter complex in which the DNA bases on the transcribed strand are available for base-pairing with an incoming substrate (*74*).

Addition of nucleoside triphosphates to T3 polymerase–promoter complexes stabilizes these complexes to dissociation, as measured by acquisition of resistance to the inhibitor heparin (*35*). Addition of only GTP and ATP gives nearly full protection; this is not unexpected since T3 RNA chains start with the sequence pppGGGA and pppGGGG (*75*).

The kinetics of T7 RNA synthesis with T7 RNA polymerase show a brief lag prior to achieving a maximal rate of incorporation (*26*). This lag is not abolished at elevated template or substrate concentrations and is probably due to the slow rate of forming stable, initiated transcriptional complexes, since chain elongation by these complexes is very rapid. It is not known what the true rate-limiting step is in this process. If T7 polymerase–promoter complexes are quite unstable it may require many encounters of polymerase with promoter before binding of substrates and chain initiation leads to trapping the polymerase. In support of this notion, addition of a competing template or an inhibitor of chain initiation, at any point during the lag, leads to blocking of transcription from the first template, hence there is no commitment of polymerase or tight binding at the promoter until transcription has begun (*28*).

The rate of RNA chain elongation by both T7 and T3 RNA polymerases is between 200 and 300 nucleotides/sec at 37° (*30, 52, 70, 71*). This is almost 10 times faster than the bacterial RNA polymerase under the same conditions (*76*). The K_s values for T7 and T3 RNA synthesis have been determined by measuring the initial rate of transcription when three substrates are fixed at concentrations about $10 \times K_m$ and the fourth nucleotide is varied. Values in the range of 40–100 μM are obtained for ATP, UTP, and CTP (*26, 35, 37*); GTP gives anomalous kinetics, probably due to its

73. Giacomoni, P. (1976). *FEBS Lett.* 72, 83.
74. Chamberlin, M. (1974). *Annu. Rev. Biochem.* 43, 721.
75. Maitra, U., Jelinek, W., Yudelevich, A., Majumder, H., and Guha, A. (1980). *PNAS* 77, 3908.
76. Kassavetis, G., and Chamberlin, M. (1981). *JBC* 256, 2777.

role as a chain initiation nucleotide (*26, 44, 70*). These values probably reflect actual K_s values for chain elongation by the phage RNA polymerases, although it cannot be ruled out that the substrates have an effect on some other step of the reaction, since the assay involves extensive recycling (see Section II,A,2).

This kinetic treatment assumes a ping-pong type of reaction mechanism (*77*). It has been shown that chain elongation by bacterial RNA polymerases fits the general equation for such a mechanism, but the process of transcriptional pausing during the elongation reaction can alter the K_s values considerably (*76, 78*). Thus the K_s values obtained for *E. coli* RNA polymerase transcribing T7 DNA are much higher, in the range from 80 to 500 μM (*79*).

Little is known about the chain termination–release phase of transcription by the phage RNA polymerases. The ability of these enzymes to efficiently recycle many times during the reaction (*26, 79*) testifies that chain termination and enzyme release are highly efficient and reasonably rapid. Utilization of the strong internal termination site for the T7 and T3 polymerases must involve some kind of DNA sequence recognition process similar to that found with bacterial RNA polymerases (*48*). Reading of this site is not completely efficient and generates a class of readthrough transcripts from both class II and class III promoter sites (*29*). It is interesting that at least two T7 genes (genes 11 and 12) appear to depend on this readthrough transcription for expression (*29*).

Although T3 RNA polymerase uses T7 promoters poorly, it reads the T7 polymerase terminator site at 61% quite well (*50*). Thus for the phage RNA polymerases, as for the bacterial RNA polymerases (*80*), the recognition of termination signals may be evolutionarily conserved. This may be due to the requirement for a physical structure at the termination site rather than to a specific nucleotide sequence at which binding takes place, since the T7 phage polymerase terminator involves an inverted repeat sequence followed by a series of U residues, just as is found for bacterial RNA polymerases [Rosa and Dunn, cited in Ref. (*33*)].

III. Other Bacteriophage RNA Polymerases

A. Bacteriophage PBS2 RNA Polymerase

Not all bacteriophage-coded RNA polymerases fit the mold of the T7 and T3 enzymes. In 1972 it was reported that the growth of the *B. subtilis*

77. Rhodes, G., and Chamberlin, M. (1974). *JBC* 249, 6675.
78. Kingston, R., Nierman, W., and Chamberlin, M. (1981). *JBC* 256, 2787.
79. Maitra, U., and Huang, H. (1972). *PNAS* 69, 55.
80. Wiggs, J., Bush, J., and Chamberlin, M. (1979). *Cell* 16, 97.

phage PBS2 was unaffected by prior treatment of the host cells with rifampicin and related compounds (*81*). While T7 phage growth becomes resistant to treatment of infected cells with rifampicin after about 5 min (*82*), T7 growth absolutely depends on transcription of early genetic regions by the host RNA polymerase (*56*). This suggested that PBS2 phage might utilize a rifampicin-resistant RNA polymerase activity even in the initial stages of growth, possibly carried in the phage particle as for the enzymes in some eukaryotic viruses (*6, 7*).

With this observation in mind, Clark, Losick, and Pero (*16*) searched in extracts of PBS2-infected *B. subtilis* for an RNA polymerase activity that would transcribe PBS2 DNA in the presence of rifampicin. Such an activity was identified and purified by ammonium sulfate precipitation followed by repeated column chromatography. The final preparation contained five major polypeptide components, of MW 80,000, 76,000, 58,000, 53,000, and 48,000, respectively, as judged by SDS-polyacrylamide electrophoresis (*17*). These were present in roughly equimolar amounts, consistent with a molecule of MW 260,000 as shown by the sedimentation coefficient of 11 S. None of these peptides is present in the *B. subtilis* host RNA polymerase, and all appear to be synthesized after phage infection.

Biochemical studies of the PBS2 RNA polymerase showed that it required the 4 ribonucleoside triphosphates and Mg^{2+} for activity (Mn^{2+} was 20% as active as Mg^{2+}). The template specificity of the enzyme was notable. PBS2 DNA was the most effective template; poly(dAdT) was also active, but other, heterologous phage DNAs were used poorly or not at all. It should be added that PBS2 phage DNA contains uracil in place of thymine.

Hybridization competition experiments using RNA transcribed from PBS2 DNA *in vitro* suggested that the PBS2 RNA polymerase gives transcripts from genetic regions used late in infection. This was consistent with the kinetics of appearance of the enzyme activity and of the enzyme subunits in infected cells: activity is first seen 10–15 min after infection.

These results suggest that the PBS2 RNA polymerase purified by Clark is involved in late phage transcription. However, they leave unanswered the question of how early transcription is carried out. It was initially suggested that PBS2 might code for an RNA polymerase activity, carried in the phage particle that could account for early, rifampicin-resistant transcription. However, the PBS2 RNA polymerase components are not detected in the phage particle.

PBS2 growth is sensitive to the drug lipiarmycin, which is an inhibitor

81. Price, A., and Frabotta, M. (1972). *BBRC* 48, 1578.
82. Summers, W., and Siegel, R. (1969). *Nature (London)* 223, 1111.

of the *B. subtilis* host RNA polymerase (*83*). Furthermore, in certain lipiarmycin-resistant cells, PBS2 growth becomes sensitive to rifampicin. These results suggest that early PBS2 transcription probably does depend on the host RNA polymerase, but that some early modification may alter its rifampicin resistance characteristics. *E. coli* mutants have been reported that have such an effect and do not map in the known RNA polymerase subunits.

B. Bacteriophage N4 RNA Polymerase

Shortly after the report that PBS2 phage transcription might be independent of the host RNA polymerase, a similar result was obtained with the *E. coli* bacteriophage N4 (*13, 14*). While early and middle classes of N4 transcription are resistant to rifampicin, late N4 transcription is sensitive and requires the host cell RNA polymerase. Thus N4 growth is rifampicin sensitive (*85*).

Again, it seemed possible that an RNA polymerase activity carried in the phage particle might be involved. In this instance, that possibility was confirmed by the observation that disrupted N4 particles contain an endogenous RNA polymerase activity (*14, 86*). This activity was not affected by rifampicin and was dependent on the 4 ribonucleoside triphosphates, and Mg^{2+}, and was highly specific for N4 phage DNA. The enzyme is coded for by an N4 phage gene, which is required for N4 early transcription as shown by isolation and study of temperature sensitive N4 mutants in the polymerase gene (*14*). Subsequent studies point to the existence of a second, N4-specific RNA polymerase activity induced in infected cells (*87*), which is responsible for synthesis of N4 middle transcripts. The latter enzyme has recently been isolated and is not the same as the early, virion enzyme, but biochemical studies are still in progress on its actual structure (W. Zehring, L. Rothman-Denes, personal communication).

The N4 RNA polymerase from N4 particles was subsequently purified to homogeneity and shown to have extremely unusual properties (*15*). Preparations contain only a single polypeptide chain of MW 350,000 as measured by SDS-polyacrylamide gel electrophoresis. The sedimentation coefficient of the enzyme (9.5 S), taken with its other hydrodynamic prop-

83. Osborne, M., and Sonenshein, A. (1980). *J. Virol.* 33, 945.
84. Lathe, R., Bue, H., Lecocq, J-P., and Bautz, E. (1980). *PNAS* 77, 3548.
85. Zivin, R., Zehring, W., and Rothman-Denes, L. (1981). *JMB* 152, 335.
86. Pesce, A., Casoli, C., and Schito, G. (1976). *Nature (London)* 262, 412.
87. Falco, S., and Rothman-Denes, L. (1979). *Virology* 95, 466.

erties confirms that the active enzyme consists of a single subunit. One or two copies are present in each phage particle.

The transcriptional properties of the purified enzyme include an absolute dependence on denatured N4 DNA; native N4 DNA is not used (*15*). However, the denatured template is transcribed asymmetrically, predominantly from one end of the genome. Further studies of the N4-directed transcription systems should be of considerable interest, especially since there would seem to be a requirement for additional components in the transcription of native N4 DNA.

5

Eukaryotic RNA Polymerases

MARTIN K. LEWIS • RICHARD R. BURGESS

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